

PEPTIDE-BASED DIAGNOSTIC REAGENTS FOR SARS

FIELD OF THE INVENTION

[0001] Severe acute respiratory syndrome (SARS) is a recently discovered atypical pneumonia that has been spreading throughout the world. The illness originated in the Guangdong province of China in November 2002 and as of May 2003 areas of local transmission had appeared in Beijing, Inner Mongolia, Shanxi, Hebei, and Tianjin regions of China, in Hong Kong, Taiwan, Mongolia, Philippines, Singapore, Viet Nam, and Canada. Cases have been reported to the World Health Organization (WHO) in 31 countries in all, on five continents¹. (The superscript numbers refer to publications, which more fully describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference. The citation of each reference is found at the end of the BACKGROUND OF THE INVENTION section). SARS has the general features of starting with a fever greater than 38°C, headache, and sore throat. The incubation period for the disease is usually from 2 to 7 days, and the patients could develop a dry, nonproductive cough and shortness of breath. Death from progressive respiratory failure occurs in about 3-8% cases. Given these possible symptoms, variable patterns of reactions to the infection are usually found among SARS infected individuals². The recent isolation of coronaviruses from multiple SARS patients, the culturing and characterization of the virus, and the transmission of disease by the virus to macaques has fulfilled Koch's postulates and established SARS coronavirus (SCoV) as the agent causing the disease³. The development of safe and readily available serologic tests for SARS, based on non-biohazardous synthetic peptide antigens, will be of significant value for the control and elimination of SCoV.

BACKGROUND OF THE INVENTION

[0002] Serologic testing for infection by SCoV has been developed using enzyme-linked immunosorbant assays (ELISA) or indirect immunofluorescence assays (IFA) based on using the whole virus as the antigen. These immunoassays

detect antibodies in the mid and late stages of SARS^{4,5}, and using these whole virus antigens SCoV-specific IgG has been found to persist through week 12 post-infection⁶. Thus, serologic assays have been proven to be useful both for the diagnosis and for the epidemiological surveillance of SARS.

[0003] The major structural proteins common to coronaviruses include the spike (S), membrane (M), and nucleocapsid (N). The S, M, and N proteins are antigens that contribute to generating the host immune response⁷. Because of the low level of similarity between the predicted amino acid sequences of SCoV and other coronaviruses, comparisons between SARS coronavirus and other coronaviruses, including the 229E human coronavirus, for primary amino acid sequences does not provide insight into the antigenic sites of SCoV⁸⁻¹⁰. Accordingly, new studies are required to identify specific SCoV antigens. For example, Spike glycoprotein (S) has been well-characterized as the surface antigen of viruses of the family *Coronaviridae* that binds to the host cell receptor. The surface protein is well known to be a target for neutralizing humoral immune responses and such responses are associated with protective immunity⁸. Therefore, antibodies against the SCoV S glycoprotein in SARS patients may have a protective value. SCoV S antigens expressed by recombinant *Escherichia coli* as intact S or as large segments are being studied as serological reagents¹¹. The membrane glycoproteins (M) of coronaviruses are also prominently surface exposed and are, in general, expected to contain immunodominant epitopes that may be useful for immunoassays⁷. For example, the M protein of canine coronavirus was cloned and expressed in *Escherichia coli* and the recombinant antigen was found to be useful in an ELISA for the detection of antibodies against the canine coronavirus in dog sera¹². However, the effectiveness of the M glycoprotein of the SARS coronavirus as a diagnostic reagent remains to be demonstrated. The N protein also can be a useful subunit antigen for the detection of anti-SCoV antibodies in patient sera and has been expressed as recombinant antigens^{13, 14}.

[0004] However, the cloning of entire proteins of the SCoV is complicated and involves the use of hazardous materials as does the immunoassay methods employing whole SCoV antigen. It is desirable to utilize the advantages of synthetic

peptides over complex biological materials such as viral extracts or recombinant proteins, for the immunosorbent in immunoassays. No biohazardous materials are used in the manufacture of synthetic peptides. The peptides are defined molecules that can be easily manufactured by a reproducible chemical process. The quality can be controlled and as a result, reproducibility of the test results can be assured. With site-specific epitopes the signal-to-noise ratio is boosted and sensitivity is heightened, while specificity is optimized due to the reduction of undesirable cross-reactive epitopes. Moreover, the use of synthetic peptides eliminates the false-positive results caused by the presence of antigenic materials originating from host cells and from recombinant protein expression systems that may be co-purified with SCoV viral and recombinant proteins. The high specificity of a synthetic peptide-based immunoassay makes it useful for differentiating infections caused by different viruses having similar clinical symptoms, e.g., RSV or influenza versus SARS. The costs for producing immunoassays having synthetic peptides are relatively low in comparison to tests using virally and recombinantly-produced antigens because very small amounts of peptide are required for each test procedure and the expense of synthesizing peptides is relatively low compared to the expense of producing biologicals¹⁵⁻¹⁷. With regard to the diagnosis of SARS, the apparent genetic stability of SCoV and the conservation of epitopes across isolates⁹, favors an approach using controlled and well-defined antigens rather than complex antigens.

[0005] At present, it is difficult to produce synthetic peptide-based immunoassays. A number of algorithms have been developed to predict candidate epitopes in proteins from the amino acid sequence, but, the present state of knowledge of protein structure does not enable the precise prediction of the amino acid sequences that represent highly antigenic epitopes. Thus, the usefulness of a synthetic peptide as an antigen must be empirically established. We have used serological analysis to map epitopes within the protein antigens of infectious viruses, as an empirical approach. These include the identification of epitopes of HIV¹⁸, HCV^{19, 20}, HTLV I and II²¹, and foot-and-mouth disease virus (FMDV)²².

[0006] The development of safe, rapid, reliable, easy-to-use and readily available serologic tests for SARS, based on non-biohazardous synthetic peptide

antigens, is of significant value. Such tests can be used in diagnostics and in epidemiological surveys for the control and eventual elimination of SARS.

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BRIEF DESCRIPTION OF THE INVENTION

[0007] The present invention relates to SCoV peptides where one or more of these peptides, or segments thereof, is used as antigenic compositions for immunological applications. Such applications include use in immunoassays and/or diagnostic kits as the solid phase immunosorbent and use for the design of a SARS vaccine where such peptides may represent protective B cell epitopes.

Immunoassays and/or diagnostic kits containing one or more of these peptides, or segments thereof, are useful to identify antibodies induced by infection or by vaccination. Such tests can be used to screen for the presence of SCoV infection in the clinic, for epidemiological surveillance, and for testing the efficacy of vaccines.

[0008] One aspect of the peptides and peptide compositions of the invention is to provide a method of diagnosing SCoV infection in a patient, comprising the steps of:

- i. contacting the patient sample with one or more SCoV antigenic peptides or immunologically functional analogues thereof under conditions conducive to binding; and
- ii. measuring binding between said patient sample and said SCoV antigenic peptides or immunologically functional analogues thereof,

wherein detection of binding between said patient sample and said SCoV antigenic peptides or immunologically functional analogues thereof indicates the presence of anti-SCoV antibodies (and therefore SCoV) in said patient sample.

[0009] According to the present invention, a series of synthetic peptides representing immunoreactive regions of the SCoV spike (S) (peptides 3180b, 3180c, 3180, 3K3180c), membrane (M) (peptides 3301, 3K3301), and nucleocapsid (N) (peptides 3187b, 3187, 3189a, 3189b, 3189, 3190a, 3190b, 3K3190b, 3190) proteins each described by specific sequences, SEQ ID NOS. 1-4, 5-6, and 7-15 for S, M, and N respectively, have been identified and made by solid phase peptide synthesis. These peptides have been found to be useful for the detection of antibodies to SCoV in sera and body fluids and for the diagnosis of SARS. In addition, according to the present invention, mixtures of these peptides may be used to detect the presence of antibodies to SCoV in sera and other body fluids.

[0010] According to the present invention, a peptide composition useful as a reagent in immunoassays for the detection of antibodies to SCoV and diagnosis of SARS may be selected from a peptide from the S protein (SEQ ID NOS. 1-4), a peptide from the M protein (SEQ ID NOS. 5-6), a peptide of the N protein (SEQ ID NOS. 7-16), or a mixture thereof. (These sequences were adopted from the Tor2 isolate of SCoV²³, made available in Entrez Genomes under RefSeq accession NC_004718 and assigned GenBank accession no. AY274119, and are localized on the SCoV genome in Figs. 2 and 3). Useful compositions are represented by the amino acid sequences shown in Table 1 as well as immunologically functional analogues, mixtures, conjugates and polymers thereof.

[0011] Another aspect of these peptide compositions allows for the development of chemically synthesized immunoassay reagents that can be readily quality controlled and used to develop sensitive and accurate methods for monitoring SCoV infection.

[0012] A further aspect of the present invention also provides immunoassay test kits for the detection and diagnosis of SARS by using an antigenically effective amount of the subject peptide composition as the solid phase immunosorbent in said

test kits. The preferred immunoassay test kit format is ELISA. These immunoassays can be used as diagnostic tools to screen for the presence of SCoV infection in the clinic, to monitor antibody and viral antigen expression during SCoV infection and thereby determine correlations between the presence of specific antibodies and the prognosis of SARS in patients, and in epidemiological surveys and/or to monitor the effectiveness of a vaccination program.

BRIEF DESCRIPTIONS OF THE DRAWINGS

[0013] Fig. 1. Genomic structure of SARS coronavirus Tor2, in comparison to that of human coronavirus 229E, and the deduced structural protein sequences of Tor2, based on Entrez Genomes RefSeq accession NC_004718, also GenBank accession no. AY274119.

[0014] Fig. 2. Location of the putative M and N protein encoding sequences on the SCoV Tor2 genome and overlapping peptides, with amino acid positions, for mapping candidate antigenic epitopes found in M and N proteins.

[0015] Fig. 3. Location of the putative S protein encoding sequence on the SCoV Tor2 genome and overlapping peptides, with amino acid positions, for mapping candidate antigenic epitopes found in S protein.

[0016] Fig. 4. Distribution of antibody reactivities to antigenic S and M peptides in sera from 672 random blood donors taken from a zero seroprevalence population. Samples were tested by the 3180c + 3301 mixed peptide SCoV ELISA. Reactivities are shown as Signal/Cutoff ratios (S/C). The mean absorbance (A_{450}) for the donor sera was 0.074 ± 0.034 . The cutoff value for the mixed peptide ELISA is set as the mean $A_{450} + 6$ standard deviations.

DETAILED DESCRIPTION OF THE INVENTION

[0017] The invention provides SCoV antigenic peptides. SCoV antigenic peptides are peptides that correspond to antigenic sites of SCoV proteins. Such peptides correspond to the portion of the amino acid sequence of naturally occurring SCoV that forms an epitope for antibody recognition. In a preferred embodiment, the epitope that forms the SCoV antigenic peptide is an epitope that is typically

recognized in a SARS infection wherein the SARS-infected patient produces antibodies to the epitope. Such epitopes can be empirically determined using samples from SARS-infected patients known to be infected with SCoV. Any immunoassay known in the art, e.g. ELISA, immunodot, immunoblot, etc., can be used to determine if antibodies are present in a sample from an SCoV-infected patient that bind to a particular fragment of a SCoV protein. In a specific embodiment, SCoV antigenic peptides are those peptides that correspond to the antigenic sites of S, M, or N proteins of SCoV. The SCoV antigenic peptides of the invention can vary in length from about 15 to about 100 amino acid residues. Preferably the SCoV antigenic peptides of the invention are about 20 to about 85 amino acid residues. In preferred embodiments, SCoV antigenic peptides are selected from the group consisting of SEQ ID NOS: 1, 5, 7, 9, and 12. The production and use of the SCoV antigenic peptides of the invention are within the scope of the present invention.

[0018] The invention further provides immunologically functional analogues of SCoV antigenic peptides. An immunologically functional analogue has been modified when compared to the corresponding of SCoV antigenic peptide in some way (e.g., change in sequence or charge, covalent attachment to another moiety, addition of one or more branched structures, and/or multimerization) yet retains substantially the same secondary and tertiary structure and/or immunogenicity as the original SCoV antigenic peptide. Thus antibodies that bind to a particular SCoV antigenic peptide will also bind to the immunologically functional analogue of that SCoV antigenic peptide with substantially similar efficacy. In preferred embodiments, immunologically functional analogues of SCoV antigenic peptides are selected from the group consisting of SEQ ID NOS: 2, 3, 4, 6, 8, 10, 11, 13, 14, and 15. The production and use of such peptide analogues are within the scope of the present invention.

[0019] In one embodiment, immunologically functional analogues of SCoV antigenic peptides are meant to encompass of SCoV antigenic peptides that have been modified by sequence substitutions, additions, or deletions. In a specific embodiment, immunologically functional analogues can be modified by sequence

substitutions that are conservative. Conservative substitutions are when one amino acid residue is substituted for another amino acid residue with similar chemical properties. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine; the polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; the positively charged (basic) amino acids include arginine, lysine and histidine; and the negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Non-conservative substitutions are when one amino acid residue is substituted for another amino acid residue that different chemical properties. Substituted residues can be either classical or non-classical amino acids. As used herein, classical amino acids are the 20 amino acids commonly found in proteins (i.e., alanine, aspartic acid, asparagine, arginine, cysteine, glycine, glutamine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan and valine) and include both the D- and L- forms of such amino acids. As used herein non-classical amino acids include both D- and L- forms of any other amino acids that can be incorporated into a protein or peptide, whether found in nature or whether synthetically produced. Non-classical amino acids include, but are not limited to, β -alanine, ornithine, norleucine, norvaline, hydroxyproline, thyroxine, gamma-amino butyric acid, homoserine, citrulline, α -amino isobutyric acid, 4-aminobutyric acid, sarcosine, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, and the like.

[0020] In another specific embodiment, immunologically functional analogues can be modified by amino acid additions to the N-terminus, C-terminus, and/or middle of the peptide. In preferred embodiments, additions are to the N-terminus or C-terminus of the peptide. Additions can be of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid residues. Such additions may constitute amino acid sequences that are present in SCoV in their entirety or in part. In a preferred embodiment, additions of amino acid sequences that are present in SCoV are of 15 amino acids or less. Such additions may also constitute amino acid sequences which are not present in SCoV. Addition of sequences which are not present in SCoV include, but are not limited to, small charged sequences (e.g.,

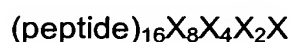
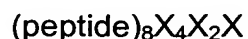
lysine-lysine-lysine) and sequences that enable the formation of branched structures (e.g., lysine or methionine). In a preferred embodiment, additions of amino acid sequences that are not present in SCoV are of 5 amino acids or less. Residue additions can be either classical or non-classical amino acids or a mixture thereof.

[0021] In another specific embodiment, immunologically functional analogues can be modified by amino acid deletions to the N-terminus, C-terminus, and/or middle of the peptide. In preferred embodiments, deletions are to the N-terminus or C-terminus of the peptide. Deletions can be of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid residues. In a preferred embodiment, deletions of amino acid sequences are of 10 amino acids or less.

[0022] In another embodiment, immunologically functional analogues of SCoV antigenic peptides are meant to encompass SCoV antigenic peptides that have been modified by covalent attachment to another moiety. The covalent attachment between the peptide and moiety may be direct or indirect (e.g. through a linker molecule) by methods known in the art. In a specific embodiment, the moiety is a carrier molecule (e.g. bovine serum albumin or human serum albumin). In another specific embodiment, the moiety is a red blood cell. In another specific embodiment, the moiety is a latex particle. In another specific embodiment, the moiety is a bead.

[0023] In another embodiment, immunologically functional analogues of SCoV antigenic peptides are meant to encompass SCoV antigenic peptides that have been modified by an alteration in charge. Such alteration in charge may be the result of amino acid substitutions, additions, or deletions, or the covalent attachment of a charged molecule. The alteration in charge may have the result of making the peptide more basic, more acidic, or more neutral as compared to the unmodified peptide. In a preferred embodiment, the peptide is made more basic by the addition of 1-5 lysine residues to the N-terminus or C-terminus. In a more preferred embodiment, the peptide is made more basic by the addition of 3 lysine residues to the N-terminus. In a most preferred embodiment, the immunologically functional analogue of a SCoV antigenic peptide with a altered charge such that is more basic is selected from the group consisting of 3K3180c, 3K3301, and 3K3190b (SEQ ID NOS. 4, 6, and 14, respectively).

[0024] In another embodiment, immunologically functional analogues of SCoV antigenic peptides are meant to encompass SCoV antigenic peptides that have been modified by addition of one or more branched structures. The branched peptides of the present invention are represented by one of the formulae:



wherein X is an amino acid or an amino acid (either classical or non-classical amino acids) having two amino groups and one carboxyl group, each group capable of forming a peptide bond linkage. In a preferred embodiment, X is lysine or methionine or non-classical amino acid residue analogue thereof. In a more preferred embodiment, X is lysine or a non classical amino acid analogue thereof (e.g., ornithine). Branched peptides of the invention include, but are not limited to, dimers, tetramers, octamers, and hexadecamers. In contrast, the linear peptides of this invention are represented by the formula



wherein Y is -OH or -NH₂.

[0025] In another embodiment, immunologically functional analogues of SCoV antigenic peptides are meant to encompass SCoV antigenic peptides that have been modified by multimerization. Such multimers may be linear or branched multimers. The individual peptides may be linked directly (e.g., as a fusion protein for linear multimers or with the addition of an amino acid residue having two amino groups and one carboxyl group for branched multimers) or indirectly (e.g., through the use of a linker molecule). Such multimers comprise at least two peptides and such peptides may or may not be identical to each other (e.g., multimers may comprise different SCoV antigenic peptides, different immunologically functional analogues of SCoV antigenic peptides, or a mixture of both).

[0026] In another embodiment, immunologically functional analogues of SCoV antigenic peptides are meant to encompass SCoV antigenic peptides that have been modified to include amino acid sequences from strains of SCoV other than Tor2. Such peptides contain substitutions (conservative or non-conservative), additions, and/or deletions such that regions of SCoV proteins from SCoV strains whose sequences vary from those of SCoV Tor2 are represented. Thus, strain-to-strain variation among different isolates of SCoV can be accommodated.

[0027] In another embodiment, immunologically functional analogues of SCoV antigenic peptides are meant to encompass SCoV antigenic peptides that have been modified by chemical modification, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to, reagents useful for protection or modification of free NH₂- groups, free COOH- groups, OH- groups, side groups of Trp-, Tyr-, Phe-, His-, Arg-, or Lys-; specific chemical cleavage by cyanogen bromide, hydroxylamine, BNPS-Skatole, acid, or alkali hydrolysis; enzymatic cleavage by trypsin, chymotrypsin, papain, V8 protease, NaBH₄; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

[0028] The present invention encompasses compositions of SCoV antigenic peptides, compositions of and immunologically functional analogues of SCoV antigenic peptides, and uses thereof. In embodiment, compositions of the invention may be a pure composition comprising only one type of SCoV antigenic peptide or immunologically functional analogue of an SCoV antigenic peptide. In another embodiment, compositions of the invention may be mixed compositions comprising more than one type of SCoV antigenic peptide or immunologically functional analogue of an SCoV antigenic peptide or a mixture of both SCoV antigenic peptides and immunologically functional analogues of SCoV antigenic peptides. Such pure or mixed compositions may be used in the methods of the invention. When mixed compositions are used in the methods of the invention (*e.g.*, for diagnosis of SCoV infection or detection of SCoV antibodies) the effective ratio of the peptides of the

can be readily determined by one of ordinary skill in the art. Typically, these ratios range from about 1 to about 50 on a weight basis of peptide.

[0029] SCoV antigenic peptides and immunologically functional analogues thereof were isolated by finding antigenic sites of relevant immunogenicity using serological analysis of overlapping synthetic peptides taken from SCoV protein antigens (Tor2 isolate of SCoV²³) with sera from patients with clinically diagnosed SARS. This process of serological validation led to the identification and further definition of SARS-immunoreactive peptides.

[0030] Accordingly, SCoV antigenic peptides and immunologically functional analogues thereof of SEQ ID NOS:1-15 were isolated. In preferred embodiments, peptides of this invention are those of SEQ ID NOS: 2, 4, 5, 6, 13, 14, and 15 (These sequences were taken from the Tor2 isolate of SCoV²³ and are positioned on the SCoV genome in Figs. 2 and 3). All the peptides of the invention are designated by their respective sequence identification numbers as shown in Table 1.

TABLE 1
Amino acid sequences of SCoV antigenic peptides derived from S, M, and N proteins

<u>S Protein- Derived Peptides</u>		
⁷⁷⁷ KYFGGFNFSQLPDPLKPTKRSFIEDLLFNKVTADAGFMKQYGE ⁸²¹	3180b	(SEQ ID No.1)
⁷⁶⁷ VKQMYKTPTLKYFGGFNFSQLPDPLKPTKRSFIEDLLFNKVTADAGFMKQYGE ⁸²¹	3180c	(SEQ ID No.2)
KKVKQMYKTPTLKYFGGFNFSQLPDPLKPTKRSFIEDLLFNKVTADAGFMKQYGE ⁸²¹	3K3180c	(SEQ ID No.3)
⁷⁵² AAEQDRNTREVFQVKQMYKTPTLKYFGGFNFSQLPDPLKPTKRSFIEDLLFNKVTADAGFMKQYGE ⁸²¹	3180	(SEQ ID No.4)
<u>M Protein- Derived Peptides</u>		
¹ MADNGTITVEELKQLLEQWNLV ²²	3301	(SEQ ID No.5)
KKK ¹ MADNGTITVEELKQLLEQWNLV ²²	3K3301	(SEQ ID No.6)
<u>N Protein- Derived Peptides</u>		
¹⁶¹ QLPQGTTLPGFYAEGSRGGSQASSRSSRSRGNSTPGSSRGNSPARMASGGGETALALLL ²²⁵	3187b	(SEQ ID No.7)
¹⁴⁶ HIGTRNPNNAATVLQLPQGTTLPGFYAEGSRGGSQASSRSSRSRGNSTPGSSRGNSPARMASGGGETALALLL ²²⁵	3187	(SEQ ID No.8)
³⁰⁶ AQFAPSASAFFGMSRIGMEVTPSGTWLTYHGAIKLDDKDPQFKDNVILLN ³⁵⁵	3189a	(SEQ ID No.9)
²⁹¹ DLIRQGTDYKHWPQIAQFAPSASAFFGMSRIGMEVTPSGTWLTYHGAIKLDDKDPQFKDNVILLN ³⁵⁵	3189b	(SEQ ID No.10)
²⁷⁶ GRRGPEQTQGNFGDQLIRQGTDYKHWPQIAQFAPSASAFFGMSRIGMEVTPSGTWLTYHGAIKLDDKDPQFKDNVILLN ³⁵⁵	3189	(SEQ ID No.11)
³⁷¹ KKDKKKKTDEAQLPQRQKKQPTVTLLPAADMDDFSRQLQNSMSGASADSTQ ⁴²²	3190a	(SEQ ID No.12)
³⁵⁶ KHIDAYKTFFPTEPKDKKKKTDEAQLPQRQKKQPTVTLLPAADMDDFSRQLQNSMSGASADSTQ ⁴²²	3190b	(SEQ ID No.13)
KKK ³⁵⁶ KHIDAYKTFFPTEPKDKKKKTDEAQLPQRQKKQPTVTLLPAADMDDFSRQLQNSMSGASADSTQ ⁴²²	3K3190b	(SEQ ID No.14)
³⁴¹ DDKDPQFKDNVILLNKHIDAYKTFFPTEPKDKKKKTDEAQLPQRQKKQPTVTLLPAADMDDFSRQLQNSMSGASADSTQ ⁴²²	3190	(SEQ ID No.15)

[0031] To identify the highly antigenic Spike (S) peptides (SEQ ID NOS 1-4), more than 100 overlapping peptides with lengths from 20 to 77 residues were designed, synthesized and tested with a panel of SARS patient and normal human sera. Among the S peptides tested, only four were found to have significant SCoV antigenicity (SEQ ID NOS. 1-4). These include a segment of 54 amino acids (SEQ ID NO: 2, 3180c). A deletion from the N-terminus of 10 amino acids resulted in peptide 3180b (SEQ ID NO: 1) having slightly diminished reactivity from 3180c (SEQ ID NO. 2). Antigenicity was also retained with an extension from the N-terminus of 3180c (SEQ ID NO. 2) of 15 amino acids (SEQ ID NO. 4, 3180) (see Table 6). Therefore, immunologically functional analogues of the subject peptides include extension on their termini by segments from the SCoV antigens of about 15 amino acids and deletions of about 10 amino acids.

[0032] By way of a non-limiting example, immunologically functional analogues of the peptides of the invention can have from 1 to about 5 additional amino acids (classical and non-classical) added to the terminal amino acids. For example, the sequence KKK (Lys-Lys-Lys) can be added to the amino terminus of any of these peptides for a change in charge, e.g., 3K3180c, 3K3301, and 3K3190b (SEQ ID NOS. 3, 6, and 14, respectively). A methionine residue can be placed at the carboxyl terminus of the peptide moiety, i.e. between the peptide moiety and a branch structure, to enable the formation of a branched structure.

The peptides of the invention are useful for the detection of SCoV antibodies in patient samples for the diagnosis of SARS. As used herein, a patient sample is meant to encompass any bodily fluid or tissue that may contain antibodies, including, but not limited to, blood, serum, plasma, saliva, urine, mucus, fecal matter, tissue extracts, tissue fluids. As used herein, the term patient is meant to encompass a mammal such as a non-primate (e.g., cow, pig, horse, cat, dog, rat etc.) and a primate (e.g., monkey and human), most preferably a human. The peptides of the invention can be used in immunoassays to detect the presence of anti-SCoV antibodies in the patient sample. Any immunoassay known in the art can be used. For example, the patient sample can be contacted with one or more SCoV

antigenic peptides or immunologically functional analogues thereof under conditions conducive to binding. Any binding between said patient sample and said SCoV antigenic peptides or immunologically functional analogues thereof can be measured by methods known in the art. Detection of binding between said patient sample and said SCoV antigenic peptides or immunologically functional analogues thereof indicates the presence of SCoV in said patient sample. In a more specific embodiment, an ELISA immunoassay can be used to assay a patient sample for the presence of anti-SCoV antibodies comprising the steps of:

- i. attaching a peptide selected from the group consisting of SEQ ID NOS: 1-4, 5-6 and 7-15 to a solid support,
- ii. exposing said peptide attached to said solid support to a sample containing antibodies from a patient sample, under conditions conducive to binding of the antibody to the peptide, and
- iii. detecting the presence of antibodies bound to said peptide attached to said solid support.

[0033] To determine the efficacy of the subject peptides in detecting anti-SCoV antibodies, the peptides are tested for their immunoreactivity with serum/plasma specimens obtained from patients with clinically diagnosed SARS. Such SARS-specific sera were provided by National Taiwan University Hospital in Taipei and by the SARS emergency hospital in Beijing, Xiaotangshan Hospital.

[0034] The peptides can be readily synthesized using standard techniques, such as the Merrifield solid phase method of synthesis and the myriad of available improvements on that technology^{24, 25}. The peptides can also be made using recombinant DNA technology. As such, nucleic acid molecules encoding the SCoV antigenic peptides and immunologically functional analogues of the SCoV antigenic peptides and compliments thereof are encompassed by the invention. Vectors, especially expression vectors, comprising the nucleic acid molecules of the invention are also encompassed by the invention. Host cells containing the vectors of the invention are also encompassed by the invention. The invention also encompasses methods of producing the SCoV antigenic peptides and immunologically functional

analogues of the SCoV antigenic peptides comprising incubating a host cell containing an expression vector comprising a nucleic acid molecule encoding an SCoV antigenic peptide and/or immunologically functional analogue of an SCoV antigenic peptide under such conditions that the SCoV antigenic peptide and/or immunologically functional analogue of an SCoV antigenic peptide is expressed.

[0035] The peptide compositions of the present invention are a significant advantage over the virus, virus-lysate and recombinant antigens of the prior art. It has been found that the SCoV is genetically stable and the conserved across isolates.⁹ An approach using controlled and well-defined immunogens rather than complex immunogens has substantial advantages. The quality of antigens produced by a chemical process or recombinant DNA technology can be better controlled and, as a result, reproducibility of the test results can be assured. No biohazardous materials are used in the manufacture of peptide antigens, reducing risks and eliminating the need for expensive biological containment. As site-specific epitopes presenting high molar concentrations of selected epitopes, signal-to-noise ratio is boosted and sensitivity is heightened by the peptide compositions, while specificity is optimized due to the reduction of undesirable cross-reactive epitopes. In a preferred embodiment, the peptides of the invention are synthesized. The use of synthetic peptides eliminates the false-positive results caused by the presence of antigenic materials originating from host cells and from recombinant protein expression systems that may be co-purified with SCoV viral and recombinant proteins. For example, sera from normal patients may have antibodies to SCoV host cells, or to recombinant *Escherichia coli*, yeast or baculovirus which are then cross-reactive with the antigenic materials used in diagnostic tests based on the biologically-derived antigens. The high specificity of the synthetic peptide-based immunoassay of the present invention makes it useful for differentiating infections caused by different viruses having similar clinical symptoms, e.g., RSV or influenza versus SARS. Another advantage of the synthetic peptides is cost. The costs for producing immunoassays having synthetic peptides are relatively low in comparison to tests using virally and recombinantly-produced antigens because smaller amounts of peptides are required for each test procedure, and because the expense of preparing peptides is relatively low¹⁵⁻¹⁷.

[0036] The peptide compositions prepared in accordance with the present invention can be used to detect SCoV antibodies by using them in an antigenically effective amount as the antigen (e.g. solid phase immunosorbent) in immunoassay test kits. In accordance with the present invention, any suitable immunoassay format can be used with the subject peptides. Such formats are well known to the ordinarily skilled artisan and have been described in many standard immunology manuals and texts, see for example, by Harlow et al.²⁶. They include, among other well-known immunoassay formats, an enzyme-linked immunoadsorbent assay (ELISA), an enzyme immunodot assay, an agglutination assay, an antibody-peptide-antibody sandwich assay, a peptide-antibody-peptide sandwich assay. In a preferred embodiment, the immunoassay is an ELISA using a solid phase coated with the above-identified peptide compositions. ELISA techniques are well known in the art.

[0037] The immunoassays and/or diagnostic kits of the present invention are used to screen patient samples (e.g., body fluids and tissues) for the presence of anti-SCoV-reactive antibody. Immunoassays containing one or more of these peptides, or segments thereof, are useful to identify antibodies induced by infection or by vaccination. Thereby, such tests can be used as diagnostic tools to aid in diagnosis of SARS, to monitor antibody and viral antigen expression during SCoV infection and thereby determine correlations between the presence of specific antibodies and the prognosis of SARS in patients, and in epidemiological surveys and/or to monitor the effectiveness of a vaccination program.

[0038] Preferably the kit of this invention is an ELISA or an agglutination test kit for detection of SCoV antibodies and thereby diagnosis of SARS. For an ELISA or an agglutination test kit, the kit contains (a) a container (e.g., a 96-well plate) having a solid phase coated with one or more of the peptides of the invention; (b) a negative control sample; (c) optionally, a positive control sample; (d) specimen diluent and (e) antibodies to species-specific (e.g., human) IgG, or protein A, protein G or protein A/G recombinants known to be reactive with all types or subtypes of immunoglobulins from multiple species, which protein is labeled with a reporter molecule. If the reporter molecule is an enzyme, then the kit also contains a substrate for said enzyme.

[0039] In an exemplified use of the subject kit, a patient sample to be tested is diluted in sample diluent if necessary and then contacted with one or more peptides of the invention for a time and under conditions for any antibodies, if present, to bind to the peptide contained in the container. After removal of unbound material (e.g., by washing with phosphate buffered saline), the secondary complex is contacted with labeled antibodies to species-specific IgG or labeled protein A, protein G, or protein A/G. These antibodies or proteins A, G or A/G bind to the secondary complex to form a tertiary complex and, since the second antibodies or proteins A, or G or A/G are labeled with a reporter molecule, when subjected to a detecting means, the tertiary complex is detected. The reporter molecule can be an enzyme, radioisotope, fluorophore, bioluminescent molecule, chemiluminescent molecule, biotin, avidin, streptavidin or the like. For ELISA the reporter molecule is preferably an enzyme.

[0040] The examples serve to illustrate the present invention and are not to be used to limit the scope of the invention.

EXAMPLE 1

Site-specific Serology for Mapping SCoV Protein Antigenic Epitopes

[0041] The first genomic sequence of a SCoV was for Tor2, isolated in Toronto²³. The deduced protein sequences of Tor2 as shown in Figure 1 were used to align the structural protein sequences of all other SCoV isolates which are available from the GenBank database. Such alignments allow for the identification of isolate-to-isolate mutations which may have occurred in the individual proteins.

[0042] The information obtained from Tor2 was used to design candidate peptide antigens as shown in Figures 2 and 3, with peptide codes from 3171 on, for identification and location of antigenic sites within SCoV structural proteins for the development of SARS diagnostic tests for antibody detection and vaccines.

[0043] Over 200 short and long peptides with sequences derived from the SCoV Spike(S), Membrane(M), and Nucleocapsid (N) proteins as shown in Figure 2 and 3 were synthesized. Although predicted secondary structures were considered in the design of these overlapping peptides, emphasis was placed on an empirical

selection of candidate antigenic peptides by their immunoreactivities with sera from patients infected by SCoV.

[0044] Short peptides comprising about 20 acids were synthesized with overlaps of about 10 residues across the entire amino acid sequences for the M, N, and S proteins. These were produced by automated peptide synthesizers (AccuChem, Lexington, KY) for the mapping of continuous epitopes.

[0045] Longer peptides comprising from about 25 up to about 100 amino acids were synthesized using Applied BioSystems Peptide Synthesizer Models 430A, 431 and 433. These were made to correspond to large regions of the S and N proteins and were useful to present longer processions of continuous epitopes and for greater ability to present conformational epitopes.

[0046] Each peptide was produced by an independent synthesis on a solid-phase support, with Fmoc protection for the terminus and side chain protecting groups of trifunctional amino acids. Completed peptides were cleaved from the solid support and side chain protecting groups removed by 90% trifluoroacetic acid. Synthetic peptide preparations were characterized for correct composition by Matrix-Assisted Laser Desorption Time-Of-Flight (MALD-TOF) Mass Spectrometry and by Reverse Phase HPLC.

[0047] Antigenicities of the synthesized peptides of varied lengths were tested for initial reactivity profiles with sera from 1) patients clinically diagnosed and confirmed for SCoV infection at National Taiwan University Hospital in Taipei and at the SARS emergency hospital in Beijing, Xiaotangshan Hospital, 2) from those of healthy blood donors with zero prevalence rate for SCoV infection, collected in 2000 from Florida blood banks, 3) from patients with other known viral infections such as HCV, and 4) from patients having sera containing interfering substances, by the standard ELISA method described below in Example 2. Those with specific reactivities to sera from confirmed SARS patients were selected for further analyses. Since the volume of the sera available for use were of very small quantity, mostly in the range of less than 100 μ L, we used two SARS samples, JPR and CZS, of which we had larger volumes to carry out the initial screens for SCoV-specific reactivities.

EXAMPLE 2

ELISA Assay Method

[0048] The wells of 96-well plates were coated separately for 1 hour at 37° with 2 µg/mL of SCoV S, M, and N protein-derived peptides or mixtures thereof using 100 µL per well in 10mM NaHCO₃ buffer, pH 9.5 unless noted otherwise.

[0049] The peptide-coated wells were incubated with 250 µL of 3% by weight of gelatin in PBS in 37°C for 1 hour to block non-specific protein binding sites, followed by three washes with PBS containing 0.05% by volume of TWEEN 20 and dried. Patient sera positive for SCoV-reactive antibody by IFA and control sera were diluted 1:20, unless otherwise noted, with PBS containing 20% by volume normal goat serum, 1% by weight gelatin and 0.05% by volume TWEEN 20. One hundred microliters of the diluted specimens were added to each of the wells and allowed to react for 60 minutes at 37°C.

[0050] The wells were then washed six times with 0.05% by volume TWEEN 20 in PBS in order to remove unbound antibodies. Horseradish peroxidase-conjugated goat anti-human IgG was used as a labeled tracer to bind with the SCoV antibody/peptide antigen complex formed in positive wells. 100 µL of the peroxidase-labeled goat anti-human IgG at a pretitered optimal dilution and in 1% by volume normal goat serum, 0.05% by volume TWEEN 20 in PBS, was added to each well and incubated at 37°C for another 30 minutes.

[0051] The wells were washed six times with 0.05% by volume TWEEN 20 in PBS to remove unbound antibody and reacted with 100 µL of the substrate mixture containing 0.04% by weight 3',3',5',5'-Tetramethylbenzidine (TMB) and 0.12% by volume hydrogen peroxide in sodium citrate buffer for another 15 minutes. This substrate mixture was used to detect the peroxidase label by forming a colored product. Reactions were stopped by the addition of 100 µL of 1.0M H₂SO₄ and absorbance at 450 nm (A₄₅₀) determined.

EXAMPLE 3

Identification of Antigenic Peptides Derived from SCoV M and S Proteins

[0052] A large collection of overlapping peptides of lengths varying from 20 to 76 residues with amino acid sequences derived from SCoV Tor2 M and S proteins were designed for empirical testing by positive sera.

[0053] In another method for epitope identification, specific features of predicted secondary structure in peptides known to be antigenic are used to select peptides which are synthesized and tested for antigenicity. However, in practice, theoretical prediction of antigenic features by algorithm has proven less useful for immunoassay development than empirical analysis for serological reactivity across the entire sequence of an antigenic protein by experiment²².

[0054] Initially, the antigenicities of SCoV M and S protein-derived peptides, each with an amino acid sequence derived from the corresponding positions shown in Figs. 2 and 3, were determined with serum samples from two confirmed SARS CoV-infected patients (coded as JPR and CZS in Tables 2 and 3) for which we had access to 2mL serum each, diluted at 1:20, in the ELISA format described in Example 2. Panels of serum/plasma samples non-reactive for SCoV, coded as NSP1, 2, 3, 4, 5, 6 and 7 for the M peptide (Table 2), and NSP 4, 6, 7, 8, 14 and 15 for S peptide (Table 3) were used in parallel runs to determine the background reactivities for the respective peptides, so as to assure the low background and specificity of the selected peptides.

[0055] After analysis of the serological data, one peptide designated as 3301 (SEQ ID NO. 5) derived from the M protein (Table 2) and one designated as 3180c derived from the S protein (SEQ ID NO. 2) (Table 3) were identified as having antigenicities with anti-SCoV antibodies. The parallel testings of peptides on the wells coated at either 1, 2, 5 or 10 µg/mL confirmed the specific reactivities for both the M (SEQ ID NO. 5) and the S (SEQ ID NO. 2) peptides for serum JPR and a preferential reactivity of serum CZS with the M peptide (SEQ ID NO. 5). The absorbancies shown in Table 2 and 3 also established the optimal plate coating concentrations for 3301 and 3180c as 2 µg/mL.

[0056] Testing of the selected M and S peptides each at the optimal 2µg/mL coating condition with a larger panel of sera from patients with confirmed SARS and from the seroconversion panels of two patients exposed to SCoV with serial bleed dates of days 1-116 and 0-97 further demonstrated SARS anti-SCoV reactivity profiles for the M and S peptides (Table 4).

Table 2. Antigenicity of SCoV M protein-derived peptide by ELISA at varied peptide coating concentrations

Sample ID	A _{450nm}			
	p3301 coating concentration (SEQ ID NO. 5)			
	1 µg/mL	2 µg/mL	5 µg/mL	10 µg/mL
Blank	0.046	0.045	0.075	0.059
NRC	0.056	0.052	0.085	0.068
SARS-JPR	0.479	0.606	0.476	0.315
SARS-CZS	0.307	0.405	0.346	0.253
NSP-1	0.053	0.052	0.083	0.067
NSP-2	0.051	0.049	0.078	0.064
NSP-3	0.050	0.059	0.077	0.064
NSP-4	0.060	0.058	0.087	0.075
NSP-5	0.055	0.059	0.089	0.077
NSP-6	0.059	0.058	0.095	0.075
NSP-7	0.051	0.049	0.081	0.148

Table 3. Antigenicity of SCoV S protein-derived peptide by ELISA at varied peptide coating concentrations

Sample ID	A _{450nm}			
	3180c coating concentration (SEQ ID NO. 2)			
	1 µg/mL	2 µg/mL	5 µg/mL	10 µg/mL
Blank	0.045	0.044	0.045	0.045
NRC	0.065	0.059	0.122	0.097
SARS-JPR	0.670	0.723	1.599	1.473
SARS-CZS	0.089	0.096	0.162	0.198
NSP-4	0.070	0.059	0.095	0.092
NSP-6	0.097	0.074	0.177	0.132
NSP-7	0.072	0.062	0.160	0.197
NSP-8	0.065	0.057	0.062	0.074
NSP-14	0.061	0.066	0.177	0.175
NSP-15	0.053 2,	0.058	0.123	0.120

Table 4. Antigenicities of SCoV S, M and N protein-derived peptides p3180c, p3301, and 3190b with an enlarged sera panel for sensitivity evaluation

Sample ID	A_{450nm}		
	3180c	3301	3190b
Blank	0.051	0.048	0.047
NRC	0.096	0.069	0.070
SARS-T3	1.617	0.856	1.467
SARS-T4	1.052	1.060	1.506
SARS-T5	1.862	0.495	2.553
SARS-T6	0.289	0.844	1.180
SARS-T7	1.131	0.540	2.474
SARS-B1	0.185	0.255	0.576
SARS-B3	0.680	0.260	0.785
SARS-B9	0.172	0.177	0.881
SARS-B10	0.198	0.174	0.161
SARS-JPR	1.484	0.780	1.558
SARS-CZS	0.107	0.593	0.818
Day 0 SARS-CSG4/22	0.107	0.069	0.070
Day 6 SARS-CSG4/28	0.161	0.168	0.194
Day 16 SARS-CSG 5/8	0.702	0.080	1.259
Day 27 SARS-CSG5/19	0.465	0.497	2.516
Day 116 SARS-CSG8/26	0.343	>3	2.066
Day 0 SARS-LFJ 5/9	0.212	0.083	0.181
Day 11 SARS-LFJ 5/20	2.681	1.340	>3
Day 17 SARS-LFJ 5/26	2.159	2.651	>3
Day 38 SARS-LFJ 6/16	2.526	2.718	>3
Day 97 SARS-LFJ 8/14	2.691	2.449	>3

EXAMPLE 4

Identification of Antigenic Peptides Derived from SCoV N Protein

[0057] A large collection of overlapping peptides of lengths varying from 20 to 81 residues with amino acid sequences derived from SCoV Tor2 N protein were designed for empirical testing by positive sera.

[0058] Initially, the antigenicities of SCoV N protein-derived peptides, each with an amino acid sequence derived from the corresponding positions shown in Fig. 2, were determined with serum samples from two confirmed SCoV-infected patients (coded as JPR and CZS in Table 5) for which we had access to 2mL serum each, diluted at 1:2.5, in the ELISA format described in Example 2. A panel of serum/plasma samples non-reactive for SCoV, coded as NSP-2, 3, and 12 (Table 5), were used in parallel runs to determine the background reactivities for the respective peptides, so as to assure the low background and specificity of the selected peptides.

[0059] After analysis of the serological data, eight peptides derived from the N protein, designated as 3187b, 3187, 3189a, 3189b, 3189, 3190a, 3190b, and 3190 (SEQ ID NOS. 7-13 and 15, respectively) were identified as having antigenicities with anti-SCoV antibodies (Table 5), with peptides of the 3190 series (SEQ ID NOS. 12, 13, 15) having the most consistent reactivities.

Table 5. Antigenicities of N protein derived peptides (A_{450nm} by ELISA)

Sample ID	3187b (ID NO 7)	3187 (ID NO 8)	3189a (ID NO 9)	3189b (ID NO 10)	3189 (ID NO 11)	3190a (ID NO12)	3190b (ID NO 13)	3190 (ID NO 15)
Blank	0.046	0.047	0.048	0.050	0.047	0.049	0.046	0.047
NRC	0.120	0.125	0.067	0.065	0.065	0.063	0.065	0.104
NSP-2	0.073	0.077	0.074	0.73	0.084	0.069	0.069	0.066
NSP-3	0.071	0.076	0.071	0.63	0.061	0.062	0.058	0.061
NSP-12	0.079	0.081	0.082	0.081	0.077	0.130	0.146	0.101
SARS-JPR (1/2.5 dil)	0.093	0.125	0.303	0.246	0.227	0.634	0.732	0.579
SARS-CZS (1/2.5 dil)	0.298	0.303	0.131	0.122	0.116	0.235	0.323	0.248

[0060] Parallel ELISA testing of antigenic N peptide 3190b (SEQ ID NO. 13) coated on the wells at 1, 2, 5 and 10 µg/mL, with the SARS and normal sera further confirmed the specific reactivity of that N peptide for sera JPR and CZS (Table 6).

Table 6. Antigenicity of SCoV N protein-derived peptide 3190b by ELISA at various peptide coating concentrations

Sample ID	A_{450nm}			
	3190b coating concentration (SEQ ID NO. 13)			
	1 µg/mL	2 µg/mL	5 µg/mL	10 µg/mL
Blank	0.063	0.054	0.047	0.050
NRC	0.067	0.070	0.069	0.071
SARS-JPR (1/6 dil)	0.316	0.323	0.332	0.306
SARS-CZS (1/2 dil)	0.272	0.458	0.493	0.436
NSP-1	0.070	0.063	0.060	0.060
NSP-2	0.073	0.065	0.066	0.064
NSP-20	0.107	0.102	0.100	0.099

[0061] Testing of selected N peptide 3190b at the 2µg/mL coating condition with a larger panel of sera from patients with confirmed SARS and from the seroconversion panels of the two subjects exposed to SCoV further demonstrated SARS anti-SCoV reactivity profiles for the N peptide (Table 4).

EXAMPLE 5

Immunologically Functional Peptide Analogues having Size or Charge Modifications by Extensions or Deletions or by Addition of Lys-Lys-Lys Residues at N Terminus Are Useful in SARS Immunoassay Formulation

[0062] Two types of immunologically functional peptide analogues having 1) size or 2) charge modifications were used to illustrate their antigenicities with anti-SCoV antibodies and thus their usefulness in assay formulation.

[0063] SARS patient serum JPR was used to assess the peptide analogues varied in size from the parent S-derived peptide 3180c (SEQ ID NO. 2). Elimination of 10 amino acids from its N-terminus resulted in a peptide designated as 3180b (SEQ ID NO. 1), or extension at its N-terminus with 15 amino acids resulted in a peptide designated as 3081 (SEQ ID NO. 4) (Table 7). The three peptides were used in ELISAs at plate coating concentrations of 2 µg/mL. Both peptide analogues

demonstrated significant immunoreactivities with SARS patient serum JPR, comparable to that of parent peptide 3180c (Table 7). The negative reactivities of all three peptides with normal serum or plasma samples (NSP 9-11) further supported their specific reactivities with the anti-SCoV antibodies present in patient serum JPR, thus establishing their status as immunologically functional analogues.

Table 7. Antigenicities of size modified analogues of S protein derived antigenic peptide p3180c

Sample ID	A _{450nm}		
	3180b	3180c	3180
Blank	0.044	0.044	0.045
NRC	0.071	0.104	0.053
SARS-JPR	1.000	1.134	0.452
SARS-CZS	0.063	0.084	0.06
NSP-SP9	0.068	0.069	0.051
NSP-10	0.059	0.061	0.058
NSP-11	0.061	0.057	0.063

⁷⁵²IAAEQDRNTREVFAQ⁷⁶⁶ // ⁷⁶⁷VKQMYKTPTL⁷⁷⁶ // ⁷⁷⁷KYFGGFNFSQILPDPPLKPTKRSF⁷⁹⁹ // ⁸⁰⁰IEDLLFNKVTLADAGFMKQYGE⁸²¹ 3180 (70-mer)
 _____ 3180b
 _____ 3180c
 _____ 3180

[0064] SARS patient samples JPR and CZS then were used to evaluate charge-modified peptide analogues to parent S and M peptides 3180c and 3301 (SEQ ID NOS. 2 and 5). Three consecutive lysine residues were added to the N-termini of peptides 3180c and 3301 resulting in peptides with designations as 3K3180c and 3K3301 (SEQ ID NOS. 4 and 6). ELISA plates were coated with each of these peptides at a concentration of 2 µg/mL and sera were used for the ELISA testing at a final 1:50 dilution, so as to illustrate the improved antigenicities of these charged analogues. As shown in Table 8, reactivity to JPR was significantly increased when comparing peptide 3301 to its further charged analogue 3K3301 (SEQ ID NO. 6), and in the comparison of peptide 3180c to its charged analogue 3K3180c (SEQ ID NO. 4). Reactivity to CZS was significantly increased for 3K3301 (SEQ ID NO. 6) when compared to its parent 3301, while low background reactivities with normal serum/plasma samples were maintained. Thus, the further charged analogues retained high specificities for anti-SCoV antibodies despite increased sensitivities and are immunologically functional analogues to their parent peptides.

Table 8. Antigenicities of charge modified (+3K) analogues of the S and M protein-derived peptides 3180c and 3301

Sample ID	A _{450nm}			
	3301	3K3301	3180c	3K3180c
Blank	0.048	0.045	0.048	0.044
NRC	0.059	0.053	0.063	0.072
SARS-JPR	0.287	0.452	0.319	0.696
SARS-CZS	0.168	0.261	0.057	0.068
NSP-6	0.059	0.052	0.062	0.079
NSP-7	0.054	0.052	0.056	0.055
NSP-12	0.067	0.058	0.072	0.097
NSP-30	0.058	0.056	0.075	0.075

EXAMPLE 6

Peptide compositions having a mixture of antigenic SCoV peptides in assay formulation enhances sensitivity

[0065] Although early detection of SARS is done by laboratory criteria such as RT-PCR assays using molecular probes and by clinical criteria such as elevated body temperature, non-productive cough, etc.², an antibody detection assay that is both sensitive and specific is desirable for serological surveillance. In developing our SARS antibody detection assays for serosurveillance and diagnosis, assay specificity had been stressed as a high priority. High specificity is a requisite of an acceptable SARS antibody test so as not to misdiagnose patients for unnecessary isolation, and to avoid the unnecessary implementation of emergency public health measures to contain an outbreak. As shown in previous examples, two selected SCoV peptides 3301 and 3180c both demonstrated reactivities with high stringency for SARS patient sera. However, an acceptable immunoassay for serosurveillance and diagnosis must also have high sensitivity. Therefore, mixtures of the antigenic S and M peptides were evaluated as antigens for complimentary sensitivity for antibody detection. Peptides 3180c and 3301 were coated alone at the previously established optimal 2 µg/mL concentration (Tables 2 and 3) and compared to peptide mixtures coated at respective concentrations of 1 + 1 µg/mL, 1.5 + 3 µg/mL, and 2 + 2 µg/mL by evaluation with SARS patient sera JPR and CZS diluted 1:20. For the 1.5 + 3 µg/mL mixed peptide coating concentration, additional SARS patient sera T3, T4, T5, T6 and T7 were also evaluated for subtle differences in assay sensitivity. As shown in Table 9, SARS patient samples CZS, T5 and T6 were found to be below the detection level with wells coated with either peptide 3180c or 3301 alone. Significant increases in antibody reactivities for these sera were observed when they were tested in wells coated with the mixed peptides, as shown by the areas marked in gray in Table 9. Moreover, the use of the peptide mixtures did not result in loss of specificity as shown by the low background reactivities of the mixtures for the normal sera. Thus sensitivity was improved and specificity was retained for the assay formulation using a mixture of complimentary peptide antigens as the solid phase antigen adsorbent.

Table 9. Improved sensitivity by using a mixture of antigenic peptides

Sample ID	A_{450nm}				
	3180c	3301	3180c+3301 coating concentration		
	coating concentration 2 μ g/mL	coating concentration 2 μ g/mL	1 + 1 μ g/mL	1.5 + 3 μ g/mL	2 + 2 μ g/mL
Blank	0.045	0.045	0.045	0.047	0.045
NRC	0.063	0.052	0.059	0.060	0.071
SARS-T3	0.456	0.426	N/A	0.525	N/A
SARS-T4	0.556	0.529	N/A	0.870	N/A
SARS-T5	1.022	0.230	N/A	0.558	N/A
SARS-T6	0.184	0.441	N/A	0.366	N/A
SARS-T7	0.518	0.277	N/A	0.379	N/A
SARS-JPR	1.030	0.606	0.622	0.723	0.991
SARS-CZS	0.161	0.405	0.314	0.271	0.415
NSP-4	N/A	N/A	0.066	N/A	0.074
NSP-6	N/A	N/A	0.071	N/A	0.088
NSP-7	N/A	N/A	0.058	N/A	0.074
NSP-8	N/A	N/A	0.054	N/A	0.055
NSP-13	N/A	N/A	0.063	N/A	0.067
NSP-14	N/A	N/A	0.062	N/A	0.068
NSP-15	N/A	N/A	0.054	N/A	0.064
NSP-17	0.063	0.053	N/A	0.065	N/A
NSP-18	0.083	0.068	N/A	0.076	N/A
NSP-19	0.070	0.062	N/A	0.061	N/A
NSP-20	0.060	0.058	N/A	0.058	N/A
NSP-21	0.071	0.064	N/A	0.068	N/A

EXAMPLE 7

Evaluation of SARS Enzyme Immunoassay in infected, random blood donor, and other non-SCoV infected populations, in a large scale analysis

[0066] Characterization of confirmed SARS patient sera. Sera from patients confirmed as having SCoV infection were shown to have antibody titers of from 1:200 to 1:800 against SCoV as determined by an immunofluorescence assay (IFA) on cells from a monkey kidney cell line infected with SCoV.

[0067] Sera from patients infected with other viruses and normal sera. Sera obtained prior to 2000 from patients with other viral infections unrelated to SARS were well documented by serological markers. A panel of 672 sera from normal blood donors was obtained from a Florida Blood bank in 2000. The seroprevalence

rate for reactivity to SCoV in these sera panels, collected at least three years prior to the report of any known SARS cases were used to evaluate the specificity of the SARS ELISA.

[0068] Analysis by a mixed peptide-based SARS SCoV ELISA. SCoV peptide ELISAs were conducted on 96-well microtiter plates coated with a mixture of SCoV S (3180c) and M (3301) peptides at 0.5 µg/mL and 3 µg/mL respectively, and with sera diluted 1:20 by the method described in Example 2.

[0069] Criteria for interpretation: Significant reactivity in the ELISA format, i.e., the cutoff value, was scored by A_{450} absorbances which were greater than the mean A_{450} plus six standard deviations of the distribution of sera from the normal population.

[0070] Results: The samples from a panel of 672 normal plasma and serum samples with a presumed zero seroprevalence rate were tested at 1:20 dilutions to assess their respective reactivities in the mixed peptide SCoV ELISA. The normal donor samples gave a mean A_{450} of 0.074 ± 0.0342 (SD), establishing a cutoff value of A_{450} 0.279. The distribution of the Signal to Cutoff (S/C) ratio for the normal sera is plotted as shown in Figure 4 with the peak S/C ratio having a value of 0.3, with none of the samples showing positive reactivity. Thus, the specificity of this ELISA on the normal samples was 100% at the set cutoff value.

[0071] The SCoV ELISA was further evaluated for specificity by testing with a large panel of samples from patients with infections unrelated to SCoV, such as HIV-1, HIV 2, HCV, HTLV 1/II, and syphilis, and with normal serum samples spiked with interference substances. These samples all tested negative by the SCoV ELISA, indicating further the high specificity of the mixed peptide test (Tables 10 and 11).

Table 10. Evaluation of the specificity of a mixed peptide SCoV ELISA for samples of HCV, HIV, HTLV, and Syphilis-infected sera and normal sera

Sample ID	A_{450nm}	S/C ratio
Blank	0.045	0.17
NRC	0.069	0.26
Cutoff	0.269	1.00
HCV pnl-1	0.071	0.26
HCV pnl-2	0.091	0.34
HCV pnl-3	0.068	0.25
HCV pnl-4	0.068	0.25
HCV pnl-5	0.099	0.37
HCV pnl-6	0.068	0.25
HCV pnl-7	0.055	0.20
HCV pnl-8	0.063	0.23
HCV pnl-9	0.072	0.27
HCV pnl-10	0.072	0.27
HCV pnl-11	0.064	0.24
HCV pnl-12	0.067	0.25
HIV 1 pnl-1	0.144	0.54
HIV 1 pnl-2	0.084	0.31
HIV 1 pnl-3	0.048	0.18
HIV 1 pnl-4	0.095	0.35
HIV 1 pnl-5	0.072	0.27
HIV 1 pnl-6	0.078	0.29
HIV 1 pnl-7	0.054	0.20
HIV 1 pnl-8	0.068	0.25
HIV 1 pnl-9	0.087	0.32
HIV 1 pnl-10	0.119	0.44
HIV 1/2 pnl-1	0.061	0.23
HIV 1/2 pnl-2	0.077	0.29
HIV 1/2 pnl-3	0.076	0.28
HIV 1/2 pnl-4	0.065	0.24
HIV 1/2 pnl-5	0.077	0.29
HIV 2 pnl-1	0.058	0.22

Sample ID	A_{450nm}	S/C ratio
HIV 2 pnl-2	0.068	0.25
HIV 2 pnl-3	0.071	0.26
HIV 2 pnl-4	0.070	0.26
HIV 2 pnl-5	0.063	0.23
HIV 2 pnl-6	0.066	0.25
HIV 2 pnl-7	0.065	0.24
HTLV I/II pnl-1	0.047	0.17
HTLV I/II pnl-2	0.049	0.18
HTLV I/II pnl-3	0.053	0.20
HTLV I/II pnl-4	0.047	0.17
HTLV I/II pnl-5	0.048	0.18
HTLV I/II pnl-6	0.049	0.18
HTLV I/II pnl-7	0.047	0.17
HTLV I/II pnl-8	0.090	0.33
HTLV I/II pnl-9	0.090	0.33
HTLV I/II pnl-10	0.058	0.22
HTLV I/II pnl-11	0.056	0.21
HTLV I/II pnl-12	0.056	0.21
Syphilis pnl-1	0.058	0.22
Syphilis pnl-2	0.079	0.29
Syphilis pnl-3	0.052	0.19
Syphilis pnl-4	0.064	0.24
Syphilis pnl-5	0.049	0.18
Syphilis pnl-6	0.058	0.22

Table 11. Evaluation of the specificity of a mixed peptide SCoV ELISA on samples with interfering substances

Sample ID	OD _{450nm} by ELISA	S/C ratio
Blank	0.045	0.17
NRC	0.066	0.25
Cutoff	0.266	1.00
interference FD2-2312-1a	0.047	0.17
interference FD2-2312-1b	0.061	0.23
interference FD2-2312-1c	0.046	0.17
interference FD2-2312-1d	0.061	0.23
interference FD2-2312-2a	0.087	0.32
interference FD2-2312-2b	0.056	0.21
interference FD2-2312-2c	0.052	0.19
interference FD2-2312-3a	0.051	0.19
interference FD2-2312-3b	0.050	0.19
interference FD2-2312-3c	0.054	0.20
interference FD2-2312-3d	0.054	0.20
interference FD2-2312-3e	0.044	0.16
interference FD2-2312-4a	0.045	0.17
interference FD2-2312-4b	0.062	0.23
interference FD2-2312-4c	0.070	0.26
interference FD2-2312-4d	0.086	0.32
interference FD2-2312-4e	0.053	0.20
interference FD2-2312-5a	0.050	0.19
interference FD2-2312-5b	0.051	0.19
interference FD2-2312-5c	0.052	0.19
interference FD2-2312-5d	0.048	0.18
interference FD2-2312-5e	0.050	0.19
interference FD2-2312-6a	0.051	0.19
interference FD2-2312-6b	0.056	0.21
interference FD2-2312-6c	0.055	0.20

Sample ID	OD _{450nm} by ELISA	S/C ratio
interference FD2-2312-6d	0.052	0.19
interference FD2-2312-6e	0.057	0.21
interference FD2-2312-7a	0.047	0.17
interference FD2-2312-7b	0.047	0.17
interference FD2-2312-7c	0.053	0.20
interference FD2-2312-7d	0.056	0.21
interference FD2-2312-7e	0.050	0.19
interference FD2-2312-8a	0.055	0.20
interference FD2-2312-8b	0.049	0.18
interference FD2-2312-8c	0.049	0.18
interference FD2-2312-8d	0.050	0.19
interference FD2-2312-8e	0.052	0.19
interference FD2-2312-9a	0.054	0.20
interference FD2-2312-9b	0.059	0.22
interference FD2-2312-9c	0.051	0.19
interference FD2-2312-9d	0.053	0.20

(a: Normal serum, b: Heparin, c: EDTA, d: ACD, and e: CPDA-1)

Table 12. Evaluation of the sensitivity of the mixed peptide SCoV ELISA on serum samples from SARS patients including two seroconversion panels

Sample ID		A _{450nm}	S/C ratio
Blank		0.045	0.17
NRC		0.069	0.26
Cutoff		0.269	1.00
SARS-JPR		1.656	6.16
SARS-CZS		1.750	6.51
SARS-T3		1.712	6.36
SARS-T4		1.806	6.71
SARS-T5		1.208	4.49
SARS-T6		1.214	4.51
SARS-T7		1.080	4.01
SARS-B1		0.309	1.15
SARS-B3		0.613	2.28
SARS-B9		0.464	1.72
Day 0	SARS-CSG4/22	0.068	0.25
Day 6	SARS-CSG4/28	0.163	0.61
Day 16	SARS-CSG 5/8	0.345	1.28
Day 27	SARS-CSG5/19	1.212	4.51
Day 116	SARS-CSG8/26	>3	>9.4
Day 0	SARS-LFJ 5/9	0.119	0.44
Day 11	SARS-LFJ 5/20	1.638	6.09
Day 17	SARS-LFJ 5/26	2.447	9.10
Day 38	SARS-LFJ -6/16	2.749	10.22
Day 97	SARS-LFJ 8/14	2.600	9.67

[0072] Further serological analysis with additional sera obtained from infected SARS patients reconfirmed the efficacy of the mixed peptide SCoV ELISA as depicted in Table 12. All sera obtained from patients with confirmed SARS and samples shown to have antibody titers against SCoV as detected by IFA were found to be positive by the mixed peptide SCoV ELISA. Table 12 also shows an analysis of the reactivity status of two SARS patients with serial bleed dates ranging from days 0 to 116 for patient CSG and from days 0 to 97 for patient LFJ. Results from these pedigreed seroconversion panels indicated that detectable levels of anti-SCoV M and S antibodies appeared as early as 11 and 16 days upon infection (for patient LFJ and CSG respectively). Such antibodies persisted in high titers throughout a 100 day period (Table 12).

EXAMPLE 8

Evaluation of SARS Enzyme Immunoassay in Populations Having Serological Reactivities to Other Viruses and Pneumonia-causing Pathogens Other than the SARS Coronavirus

[0073] The high specificity of the synthetic peptide-based SARS immunoassay should make it a useful serological assay for differentiating SARS from pneumonia caused by other pathogens.

[0074] The mixed peptide ELISA described and characterized above in Examples 6 and 7 was further evaluated on sera panels from National Taiwan University Medical School having samples from 1) 10 patients naturally infected with influenza (two sequential bleeds per flu patient), 2) 10 patients with rubella, 3) eight with cytomegalovirus (CMV) infection, 4) nine with Epstein Barr Virus (EBV), 5) five infected with mycoplasma, a bacterial cause for the kind of atypical pneumonia caused by SCoV, and 6) pre- and post-vaccine bleeds from 16 patients given influenza vaccine. Also included were serial samples collected from three SARS patients on the indicated days. All samples were tested in duplicate. The data (Table 13) were negative for all patients infected with pneumonia-causing pathogens other than the SARS coronavirus and were positive on all but early bleed SARS patient samples.

[0075] In addition, human coronaviruses 229E and OC43 belong to a different serotype from the SARS coronavirus, and an immunoassay of adequate specificity for distinguishing SARS from other coronavirus respiratory infections should not have cross-reactivity to these other coronaviruses^{10, 23}. The mixed peptide SARS ELISA did not detect reactivities for these coronaviruses in the 692 US blood donor samples presented in Example 7 (Figure 4). There is a strong expectation for reactivity among a US population because, even among healthy young adults, the incidence of OC43 and 229E respiratory infections is as high as 8%²⁷. The lack of detectable reactivities among a large number the US serum and plasma samples supports the specificity of the mixed peptide SARS ELISA.

[0076] The specificity and sensitivity results of this Example demonstrate that the mixed peptide SARS ELISA is an appropriate method for distinguishing pneumonia caused by SCoV from pneumonia caused by other pathogens.

[0077] In summary, a highly sensitive and specific SCoV antibody detection test in the simple, rapid, and convenient ELISA format was developed for the large scale application of serosurveillance for SARS. The test is based on a solid phase immunosorbent comprising antigenic synthetic peptides corresponding to segments of the SCoV M, S, and N proteins and immunologically functional analogues thereof, branched as well as linear forms, conjugates, and polymers. The immunoassay is suitable for use in combination with molecular probe-based or other virus detection systems. The high specificity of this peptide-based SCoV immunoassay system, provided by the high stringency imposed on the selection of the SCoV antigenic peptides, and the high sensitivity provided by the mixture of peptides having complementary site-specific epitopes, results in a test that is appropriate for national epidemiological surveys. Such tests can be used by countries suffering from SARS outbreak or suspecting the presence of SARS for look back epidemiology studies. Also, a highly specific immunoassay can be used to differentiate SCoV infection from diseases caused by unrelated respiratory viruses and bacteria. An immunoassay of the invention can eliminate the untoward over-reporting of SARS, reduce the number of patients in isolation, and reduce the other costs associated with emergency measures to contain disease transmission.

Table 13. Evaluation of the specificity of the mixed peptide SCoV ELISA on samples from patients infected with influenza, rubella, CMV, EBV, mycoplasma, or SCoV, and flu vaccine sera.

Sample ID	Serum description	S/C ratio	Reactivity
UBIAP1-T1	Flu, natural infection	0.21	neg
UBIAP1-T2	Flu, natural infection	0.20	neg
UBIAP2-T1	Flu, natural infection	0.23	neg
UBIAP2-T2	Flu, natural infection	0.22	neg
UBIAP3-T1	Flu, natural infection	0.19	neg
UBIAP3-T2	Flu, natural infection	0.19	neg
UBIAP4-T1	Flu, natural infection	0.19	neg
UBIAP4-T2	Flu, natural infection	0.30	neg
UBIAP5-T1	Flu, natural infection	0.33	neg
UBIAP5-T2	Flu, natural infection	0.20	neg
UBIAP6-T1	Flu, natural infection	0.22	neg
UBIAP6-T2	Flu, natural infection	0.21	neg
UBIAP7-T1	Flu, natural infection	0.22	neg
UBIAP7-T2	Flu, natural infection	0.23	neg
UBIAP8-T1	Flu, natural infection	0.22	neg
UBIAP8-T2	Flu, natural infection	0.21	neg
UBIAP9-T1	Flu, natural infection	0.22	Neg
UBIAP10-T1	Flu, natural infection	0.22	Neg
038074	Rubella infection	0.18	Neg
038076	Rubella infection	0.35	Neg
038092	Rubella infection	0.20	Neg
038093	Rubella infection	0.29	Neg
038095	Rubella infection	0.19	Neg
038099	Rubella infection	0.23	Neg
038100	Rubella infection	0.38	Neg
038109	Rubella infection	0.24	Neg
038112	Rubella infection	0.30	Neg
038115	Rubella infection	0.38	Neg
038009	CMV infection	0.38	Neg
038010	CMV infection	0.29	Neg
038013	CMV infection	0.32	Neg
038014	CMV infection	0.22	Neg
038043	CMV infection	0.29	Neg
038047	CMV infection	0.32	Neg
038065	CMV infection	0.52	Neg
038077	CMV infection	0.22	Neg
038045	EBV infection	0.26	Neg
038050	EBV infection	0.22	Neg
038068	EBV infection	0.33	Neg
038080	EBV infection	0.36	Neg
038082	EBV infection	0.22	Neg
038086	EBV infection	0.33	Neg
038087	EBV infection	0.25	Neg
038094	EBV infection	0.24	Neg
038105	EBV infection	0.22	Neg
036893	Mycoplasma infection	0.27	Neg

Sample ID	S rum description	S/C ratio	Reactivity
37066	Mycoplasma infection	0.19	Neg
036883	Mycoplasma infection	0.30	Neg
037893	Mycoplasma infection	0.52	Neg
037623	Mycoplasma infection	0.46	Neg
Flu vac 1-T1	Flu vaccinate pre-bleed	0.28	Neg
Flu vac 1-T2	Flu vaccinate	0.24	Neg
Flu vac 8-T1	Flu vaccinate pre-bleed	0.23	Neg
Flu vac 8-T2	Flu vaccinate	0.24	Neg
Flu vac 11-T1	Flu vaccinate pre-bleed	0.23	Neg
Flu vac 11-T2	Flu vaccinate	0.25	Neg
Flu vac 12-T1	Flu vaccinate pre-bleed	0.20	Neg
Flu vac 12-T2	Flu vaccinate	0.21	Neg
Flu vac 13-T1	Flu vaccinate pre-bleed	0.22	Neg
Flu vac 13-T2	Flu vaccinate	0.20	Neg
Flu vac 14-T1	Flu vaccinate pre-bleed	0.21	Neg
Flu vac 14-T2	Flu vaccinate	0.21	Neg
Flu vac 19-T1	Flu vaccinate pre-bleed	0.25	Neg
Flu vac 19-T2	Flu vaccinate	0.25	Neg
Flu vac 20-T1	Flu vaccinate pre-bleed	0.23	Neg
Flu vac 20-T2	Flu vaccinate	0.22	Neg
Flu vac 21-T1	Flu vaccinate pre-bleed	0.22	Neg
Flu vac 21-T2	Flu vaccinate	0.18	Neg
Flu vac 22-T1	Flu vaccinate pre-bleed	0.27	Neg
Flu vac 22-T2	Flu vaccinate	0.25	Neg
Flu vac 23-T1	Flu vaccinate pre-bleed	0.31	Neg
Flu vac 23-T2	Flu vaccinate	0.25	Neg
Flu vac 24-T1	Flu vaccinate pre-bleed	0.25	Neg
Flu vac 24-T2	Flu vaccinate	0.27	Neg
Flu vac 25-T1	Flu vaccinate pre-bleed	0.29	Neg
Flu vac 25-T2	Flu vaccinate	0.32	Neg
Flu vac 26-T1	Flu vaccinate pre-bleed	0.37	Neg
Flu vac 26-T2	Flu vaccinate	0.28	Neg
Flu vac 29-T1	Flu vaccinate pre-bleed	0.27	Neg
Flu vac 29-T2	Flu vaccinate	0.23	Neg
Flu vac 30-T1	Flu vaccinate pre-bleed	0.25	Neg
Flu vac 30-T2	Flu vaccinate	0.21	Neg
SARS-1-T1	SCoV infection 1 (5/1/03)	1.10	Pos
SARS-1-T2	SCoV infection 4 (5/2/03)	1.37	Pos
SARS-1-T3	SCoV infection 4 (5/5/03)	1.61	Pos
SARS-3-T1	SCoV infection 3 (5/7/03)	1.01	Pos
SARS-3-T2	SCoV infection 3 (5/10/03)	1.57	Pos
SARS-4-T1	SCoV infection 4 (5/5/03)	0.29	Neg
SARS-4-T2	SCoV infection 4 (5/9/03)	2.50	Pos

Values are reported as the Signal/Cutoff ratio (S/C ratio) for the mean values from each sample.

Notations "T1" and "T2" indicate serial bleeds on the same patient.